

# **APPENDIX A**

**(CLEAN VERSION OF SUBSTITUTE SPECIFICATION)**

**(Serial No. not yet assigned)**

USE OF ERYTHROPOIETIN FOR THE PREVENTIVE OR CURATIVE TREATMENT OF CARDIAC FAILURE

## FIELD OF THE INVENTION

The invention relates to the field of medicine. More in particular the present invention relates to the treatment of hypoxia related disorders in mammals and compounds and pharmaceutical preparations for use therein.

## BACKGROUND OF THE INVENTION

Cardiac failure is a chronic clinical syndrome characterized by the heart being unable to adequately pump blood throughout the body. Generally, it is caused by any disease or conditions that causes loss of cardiac tissue, especially of the left ventricle. The most common causes include cardiac infarction, coronary artery disease, myocarditis, chemotherapy, alcoholism and cardiomyopathy. On the other hand cardiac failure may be caused by diseases or conditions, which require an excessive demand for cardiac output. The most common causes include hypertension, valvular heart diseases (most often mitral insufficiency and aortic stenosis) and disorders of the thyroid gland. The long-term extra demand on the heart will lead to a compensatory hypertrophy of the cardiomyocytes. As the capillary network does not extend, hypertrophy will lead to a relative ischemia, because the diffusion pathway for oxygen will increase. Recently, the importance of the role of ischemia in cardiac failure has been put forward (Van den Heuvel et al., 2000).

Thus far, the treatment of patients suffering from ischemic heart disease and subsequent cardiac damage

leading to heart failure has focused on early  
reperfusion. Although additional cell protection therapy  
might - in theory - limit the damage that is caused by  
myocardial ischemia, and hence reduce morbidity and  
5 mortality, no sufficient therapies exist to date.  
Additional supportive therapy to protect the myocardium  
in acute ischemic conditions consists nowadays in  
administration of beta-blockers, calcium antagonists and  
nitrates. However, these therapies have a low efficacy  
10 and alternative and/or additional strategies are needed.

## BRIEF DESCRIPTION OF THE FIGURES

**Fig. 1.** Real Time RT-PCR of EPO-R mRNA. Specificity was  
15 checked with the use of restriction enzyme (NciI) for  
partial digestion of the 72 bp EPO-R product in expected  
fragments (39bp and 34 bp).

**Fig. 2.** Western blot. lane 1-3: MAPK (pERK1=44kD; pERK2  
20 =42 Kd) in sham treated hearts; lane 4-6: MAPK in EPO  
treated hearts; lane 7: EPO in sham treated heart; lane  
8: EPO-R in sham treated heart

## 25 SUMMARY OF THE INVENTION

The present invention provides for the use of  
erythropoietin (EPO), or derivatives or functional  
analogues thereof, for the preparation of a medicament  
for the preventive and/or curative treatment of patients  
30 suffering from, or at risk of suffering from cardiac  
failure. Treatment with EPO for these conditions can be  
beneficial, irrespective of their cause and nature. The  
invention also provides a method for treating a patient  
suffering from, or at risk of suffering from cardiac

failure, said method comprising a step of administering to said patient erythropoietin, or a derivative or functional analogue thereof. In one aspect of the invention, the patient suffering from heart failure is

5 not anemic. Although recent clinical studies demonstrated the beneficial effects of EPO in patients with congestive heart failure (CHF) that also had anemia (Silverberg et al., 2000 and 2001), the person skilled in the art before the present invention would not treat patients with heart

10 failure by using EPO in the absence of specific other indications for the use of EPO, such as anemia, kidney disease or leukemia. A certain fraction of CHF patients is anemic (low hematocrit/low hemoglobin percentage) and a correlation exists between the severity of the

15 condition of CHF and the degree of anemia. When patients with anemia in CHF were treated with recombinant EPO, an improvement with respect to cardiac function, renal function and a decrease in the need for diuretics and hospitalization was observed (Silverberg et al. 2000 and

20 2001). Other publications (EP0813877; Mancini et al, 2001) also describe the use of EPO to raise the red blood cells and/or prevent anemia in case of congestive heart failure. It appears that thus far, the improved condition of heart patients upon treatment with EPO was ascribed to

25 the purposeful hematocrit elevation, when patients had a medical indication to treat them with EPO, thus improving peripheral oxygenation by a mechanism unrelated to a change in cardiac function. The present invention for the first time discloses the use of EPO for the treatment of

30 heart failure irrespective of whether the hematocrit value (red blood cell count) of the patient is lower than normal or not. This provides cardiac failure per se as a novel indication for the use of EPO. The present invention therefore provides for the use of EPO for

treatment of patients with heart failure, wherein said patients do not necessarily have another indication besides heart failure, which would otherwise have warranted the treatment of such a patient with EPO based on the presently available knowledge.

In certain embodiments, the EPO, or derivative or functional analogue thereof, has been produced in a host cell expressing at least the E1A protein of an adenovirus, preferably in a host cell derived from a PER.C6<sup>TM</sup> cell.

The invention further provides erythropoietin, or a functional part, derivative and/or analogue thereof, for treatment of a patient suffering from, or at risk of suffering from a chronic and/or acute coronary syndrome. Preferably, said EPO has been recombinantly produced on a host cell that expresses at least the E1A protein of an adenovirus, more preferably on a host cell derived from a PER.C6<sup>TM</sup> cell. Although the use of EPO to protect the myocardium from acute ischemic injury has been described (see WO 00/61164, WO 01/82952), the EPO used may cause a concomitant significant increase in hematocrit values, which can be regarded as an undesired side-effect for this application. The use of EPO derived from PER.C6<sup>TM</sup>, or another E1A expressing host cell, leads to less of this side-effect and therefore is beneficial (see also PCT/NL02/00686 for the demonstration that EPO produced on PER.C6<sup>TM</sup> is functional, but gives rise to less increase in hematocrit values, when compared with a commercially available EPO preparation (EPREX)).

The invention further provides the use of erythropoietin, or derivatives or functional analogues thereof, for the preparation of a medicament for the preventive and/or curative treatment of chronic and/or acute coronary syndromes. The invention also provides

pharmaceutically effective preparations comprising EPO, or a derivative or functional analogue thereof for such treatments.

Furthermore, the invention provides methods for  
5 treating a patient suffering from, or at risk of  
suffering from, undesirable effects of chronic or acute  
coronary syndromes, comprising the steps of administering  
to the patient erythropoietin, or a derivative or  
analogue thereof, in an amount sufficient to prevent or  
10 reduce said undesirable effects. Undesirable effects that  
may be decreased and/or inhibited by the compounds of the  
present invention include detrimental effects such as  
apoptosis and/or necrosis of heart muscle cells. The  
effects on such cells most likely occur through the  
15 interaction of compounds of the invention with receptors  
present on such cells. Direct effects brought about by  
compounds of the present invention also include  
angiogenic effects, through which certain hypoxia-related  
coronary syndromes are reduced in severity, both in acute  
20 as well as in chronic cases.

#### DETAILED DESCRIPTION

Erythropoietin (EPO), EPO derivatives and functional  
25 analogues, are when appropriate hereinafter referred to  
as "EPO" for the sake of brevity. EPO is a protein well  
known for its role in differentiating hematopoietic stem  
cells into red blood cells, but it has many additional  
functions as well. This application reveals a novel EPO  
30 and EPO-receptor (EPO-R) system in the heart, which  
knowledge is converted into practical use by  
administering EPO to patients with heart failure,  
according to the present invention.

Cardiac failure, also called heart failure, or chronic heart failure or congestive heart failure, is defined as a heart disease, in which the heart is not able to pump blood at a rate required by the metabolizing  
5 tissues, or when the heart can do so only with an elevated filling pressure. Treatment of heart failure with EPO according to the invention includes treatment of patients having or being at risk of having cardiac infarction, coronary artery disease, myocarditis,  
10 chemotherapy, alcoholism, cardiomyopathy, hypertension, valvular heart diseases (most often mitral insufficiency and aortic stenosis) and disorders of the thyroid gland, and the like.

A patient according to the invention can be human,  
15 but may also include an animal with heart failure. Therefore, treatment according to the invention may pertain to humans as well as to other animal species.

A non-anemic patient as used herein, is a patient that has a hemoglobin value that is considered as being  
20 within the normal range, which value would not lead a physician to prescribe EPO to this patient. Up till now, application of EPO is restricted to the prevention or correction of anaemia in specific patient populations, including the (pre)dialysis phase of chronic renal  
25 insufficiency, cytostatic therapy, premature infants and as preparation for autologous blood transfusion or surgical procedures with anticipated major blood loss. The general aim in such cases is to increase hemoglobin levels (Hb) by increasing the number of red blood cells  
30 (hematocrit) to a specific range by adapting standard dosage regimes to individual needs. Depending on the patient population, the optimal Hb level ranges from a lower limit of 6.5-7.5 mmol/L to an upper limit of 8.0-8.7 mmol/L.

According to one aspect of the invention the EPO administered or formulated for use in the treatment of myocardial disease is EPO as may be isolated from any suitable source. Preferably, human EPO is recombinantly produced and isolated from a suitable recombinant host cell and/or from the culture medium. In the case of recombinant production, the host may suitably be chosen from any cell capable of recombinantly producing protein, such as bacterial host cells (e.g., E.coli, B.subtilis), yeast (e.g., S.cerevisiae, K.lactis), fungi (e.g., A.niger, Pichia), mammalian cells (e.g., CHO, BHK) including human cells. According to one aspect of the invention, EPO is recombinantly produced in an immortalised human cell line, in particular PER.C6™ (ECACC deposit nr. 96022940). It is also possible to administer EPO in a gene-therapy setting according to the invention, for instance by treating a patient with a vector comprising a nucleic acid sequence capable of expressing EPO when delivered to a target cell.

Derivatives of EPO refer to modifications of the source EPO, which may be urinary EPO, or EPO recombinantly producible from a cDNA or gene sequence, wherein the expression product has one or more modifications relative to the source EPO, which modifications may be in the primary structure, by substitution of one or more amino acid residues (such as in NESP), deletion, addition or relocation of one or more amino acid residues, or alterations in the post- or peritranslational modification of the protein backbone, such as hydroxylations, phosphorylations or glycosylations of amino acid residues, sulphur bridges, and the like. Derivatives also encompass naturally or non-naturally occurring EPO variants coupled to non-EPO related proteinaceous moieties or even to non-proteinaceous



moieties. Derivatives of EPO are encompassed by the instant invention, as long as they interact with the EPO receptor and cause a reduction or prevention of the undesirable effects caused by chronic or acute coronary syndromes that include but are not limited to myocardial ischemia, myocardial infarction or heart failure, or caused by hypoxia conditions in the heart in general. As a measure for the occurrence of undesirable effects, the degree of apoptosis and/or necrosis in the heart tissue may be determined and/or the levels of purines in the coronary effluent circulation, or by any other means known in the art.

Functional analogues of EPO refer to molecules not necessarily derived from naturally or non-naturally occurring EPO, that are capable of mimicking the interaction of EPO with its receptor, whereby the undesirable effects caused by chronic or acute myocardial ischemia or myocardial infarction, or hypoxia in the heart in general, are reduced and/or prevented. Such functional analogues may comprise peptidomimetics and/or non-peptidic molecules mimicking the idiotope interacting with the EPO-R. It will be understood by those of skill in the art, that the functional analogue according to the invention need not necessarily interact with the same idiotope, or in the same way, as long as its interaction mimics the interaction of EPO with its receptor. Functional analogues may suitably be screened and selected from (synthetic) peptide libraries, phage or ribosome polypeptide display libraries, or small molecule libraries. Those of skill in the art are capable of screening for, or designing functional analogues, and test their functionality in assays disclosed herein. In addition to assays based on apoptosis and/or purine determination, other methods, such as methods towards

measuring cell necrosis that are generally known in the art, may be used to test the functionality of the analogue in reducing and/or preventing the undesirable effects of hypoxia.

5 EPO may be administered to a mammal in any pharmaceutically acceptable form. Generally, EPO will be administered parenterally or subcutaneously (sc), but the way of administration may vary from time to time. Whenever it is needed to obtain a quick response, it may  
10 be desirable to add EPO in high dose form by means known to quickly deliver the pharmaceutical to the heart. Instances where this is clearly desired are, for example, where the patient suffers from acute syndromes such as acute myocardial ischemia, myocardial infarction or acute  
15 heart failure. In these circumstances, doses typically rise above the doses that are administered to human patients suffering from anemia or suffering from chronic coronary syndromes (Silverberg et al. 2000 and 2001). Normal doses that are administered to adult renal failure  
20 patients are in the range of 4000 - 7500 IU per week (80 - 100 kg body weight). These amounts are normally divided into 3 separate doses per week for the commercially available epoetin alpha or Eprex (EPO produced on CHO cells). Higher doses for the treatment of acute coronary  
25 disorders may be given daily or even more frequently. The maximum tolerable dose may have to be determined, in order to prevent hematocrit values and hemoglobin concentrations to rise too sharply. Persons of ordinary skill know how to monitor hematocrit values and  
30 hemoglobin concentrations in patients to prevent undesired side effects, such as extreme high blood pressure that may occur in later stages of the treatment. These administration schemes contrast the schemes used by Silverberg et al. (2000 and 2001) to treat anemic

patients that suffer from congestive heart failure, where administration of EPO was prolonged for weeks, or even months. For acute coronary syndromes it might not be necessary to prolong the treatment with the high doses for several months, since the protective effect is required instantly and undesired side-effects might occur when such high doses are given for prolonged periods of time. In case of chronic coronary syndromes including but not limited to myocardial ischemia or heart failure, lower doses may be administered during a longer time interval. Heart failure includes both acute heart failure syndromes, such as in the frame of myocardial infarction, but also reduced pumping of the heart in chronic cases. These applied doses are comparable to doses given to renal failure patients that suffer from the lack of EPO. Doses for non-acute hypoxia related myocardial disorders, may range from 10 to 10000 IU per administration, preferably 1000 to 2500 IU per administration (for an adult of 80 - 100 kg). Also in this case monitoring may be necessary to prevent unwanted side effects. As disclosed in WO 00/63403, EPO can also be recombinantly produced on PER.C6<sup>TM</sup> cells. It was recently described (see patent application PCT/NL02/00686) that EPO thus produced leads to a significantly lower increase of the hematocrit value upon administration than similar doses of recombinant EPO currently commercially available (EPREX). This appears mainly due to the specific posttranslational modifications of the EPO thus produced, which appear related to the presence of at least the E1A sequence of an adenovirus in expressible format in the host cell used for recombinant production of EPO. A less pronounced increase in hematocrit value upon administration of EPO is beneficial for use according to the present invention. It is therefore a preferred

embodiment of the present invention to use EPO according to the invention, whereby the EPO has been recombinantly produced in a host cell expressing at least the E1A protein, or a derivative or functional analogue thereof  
5 (see PCT/NL02/00686). Preferably, said host cell is a PER.C6<sup>TM</sup> cell. Such EPO can be used according to the invention for both chronic and acute coronary syndromes.

Novel formulations of EPO-like proteins are known in the art. The Novel Erythropoiesis Stimulating Protein  
10 (NESP) is known to be effective for longer periods of time due to its modified glycosylation pattern, which makes the administration schedule such that only once a week a dose is required to sort the effects that were formerly found with three doses a week of the original  
15 recombinant EPO protein. For the treatment of acute or chronic coronary syndromes it might also be useful to apply NESP, which should be administered in a similar way as described above for EPO, namely at higher (and possibly more frequent) doses in the case of acute  
20 coronary syndromes and at comparable (and equally frequent) doses in the case of chronic heart failure. It remains to be seen whether the modified glycosylation of NESP as compared to EPO has any differentiating effect on the EPO-R present on myocytes and endothelial cells in  
25 the blood vessels of the heart.

Pharmaceutically acceptable formulations according to the invention typically comprise EPO according to the invention, usually together with pharmaceutically acceptable excipients, diluents, solvents, and  
30 optionally, compounds acting in an additive or even synergistic fashion. Compounds of the latter category comprise compounds of the statin family, such as lovastatin, simvastatin, angiotensin converting enzyme inhibitors (ACE-inhibitors), and the like.

It is worth noting, that the protective effect of EPO according to the invention on hypoxia induced myocardial damage, as determined by purine analysis in the coronary effluent and/or the degree of apoptotic cells in the myocardium, is observed within minutes after subcutaneous administration. It is difficult to imagine that this effect should be ascribed to EPO's known stimulating effect on angiogenesis, or to its haematopoietic effect for that matter, since these effects are typically not observed within the time frame of minutes, but rather days, or even weeks. It is tempting, therefore, to speculate that the cell protective effect of EPO observed within minutes after administration is brought about by a direct intervention of EPO and tissues of, or in direct contact with the myocardium. The fact that the EPO-R is found to be expressed on the cell surface of the myocytes (as is shown in this invention) strongly suggests that direct anti-apoptotic and anti-necrotic effects occur through the action of EPO on these receptors, while the direct angiogenic effects of EPO most likely occur through the EPO-R expressed on endothelial cells in the capillaries. This effect may occur in vitro as well as in vivo.

The invention will now be illustrated by the following examples.

#### EXAMPLES

30

##### **Example 1. Detection of EPO and EPO-R in normal human and rat heart tissue.**

It has been found that EPO and the EPO-R are expressed in fetal cardiac tissue (Juul et al. 1998).

Despite the increasing body of literature on the expression of EPO and its receptor, and the putative roles associated therewith, little, if anything, is known of the distribution of EPO and EPO-R in adult heart  
5 tissue.

Expression of EPO and EPO-R was examined by real-time RT-PCR, western blotting and immunohistochemistry on rat heart tissue and by western blotting and immunohistochemistry on human heart biopsies.

10

#### **Rat heart (Langendorff set-up)**

For this, ischemic/reperfusion (I/R) experiments in isolated rat hearts suspended in a so-called Langendorff apparatus (Van Gilst et al. 1988) were performed with and  
15 without the administration of EPO, using methods generally known to persons skilled in the art.

Male Sprague Dawley Rats weighing approximately 300 grams (n=12) were divided into 4 experimental groups. Two groups received global cardiac ischemia by reducing  
20 coronary flow to 0.6 ml/min for 30 min. followed by reperfusion for 45 min. Two other groups were without ischemia. Within each of the groups half of the rats were treated with EPO (10 U/ml) and half with saline. Rats were anaesthetised and 500U of heparin was injected in  
25 the tail vein. The heart was rapidly excised and the aorta was immediately retrogradely perfused by a modified Tyrode solution (glucose 10, NaCl 128.3, KCl 4.7, NaHCO<sub>3</sub> 20.2, CaCl<sub>2</sub> 1.35, NaH<sub>2</sub>PO<sub>4</sub> 0.42, MgCl<sub>2</sub> 1.05; all mmol/liter) and was equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.  
30 Perfusion pressure was maintained at 60mmHg. Coronary flow (CF) was measured by a microprocessor, which controlled the perfusion pressure by adjusting the peristaltic perfusion pump. CF, heart rate (HR), and left ventricular peak pressure were monitored continuously.

After equilibrating for 5 minutes, hearts were perfused for 20 minutes with EPO or saline before the I/R protocol started.

5 *Real-time RT-PCR*

Total RNA was isolated from rat left ventricle and processed as described previously (Brundel et al., 1999). Briefly, cDNA was synthesized by incubating 1 µg of RNA in reverse transcription buffer, 200 ng of random hexamers  
10 with 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase, 1 mmol/L of each dNTP, and 1 U of RNase inhibitor (Promega). Synthesis reaction was performed for 10 minutes at 20°C, 20 minutes at 42°C, 5 minutes at 99°C, and 5 minutes at 4°C. All products were checked for  
15 contaminating DNA. Fragments of EPO-R were amplified (Forward primer: CAGGACACCTACCTGGTATTGGA; reverse primer: CAGGCCCGAGAGGTTCTCA , eurogentec, Belgium) with a GeneAmp® 5700 (Perkin-Elmer/ABI) employing a 40 cycle protocol consisting of 30 sec. at 94°C, 1 min at 56°C and  
20 30 sec. at 72°C, After the last cycle the 72°C elongation step was extended to 5 min. The PCR products were detected using SYBR-green I. EPO-R was detected in cardiac samples of normal rat heart tissue and in tissue subjected in vitro to a 30 min ischemic period  
25 irrespective of treatment with EPO.

To confirm specificity of the product, the amplified fragments were treated for 3 h with the restriction enzyme NciI for partial digestion and separated on 2.5% agarose gels by gel-electrophoresis and stained with  
30 ethidium bromide. Restriction analysis confirmed splicing of the obtained product in two fragments of the expected size (34 and 39 bp, Fig. 1).

In contrast to EPO-R, we were unable to detect EPO mRNA in rat heart using the real-time RT-PCR method described

by Neumcke et al. (1999) (while brain tissue was positive in the same PCR reaction).

#### *Western blotting*

5 Western blotting was performed according to standard methods (Brundel et al., 1999) on midpapillary slices from the left ventricle of rat heart, which were snap frozen in liquid nitrogen. In brief, frozen LV tissues (~50 mg) were homogenized in 1 ml of ice-cold protein  
10 lysis buffer and protease inhibitors. The homogenates were then centrifuged for 20 minutes at 4°C at 14000 rpm, and the supernatant was transferred into a clean tube and kept on ice. Protein concentration was determined by using a standard protein assay (Bio-Rad protein assay,  
15 Bio-Rad, Richmond, CA). Protein samples (50 µg) were subjected to SDS-PAGE on 7.5 % acrylamide gels, and then transferred to PVDF membranes using a wet transfer unit (for 3 hours at 100 mA). The membranes were then blocked for 20 minutes with Tris-buffered saline containing 0.04%  
20 Tween 20 plus 5% non-fat dried milk, after which they were incubated for 3 hours with the primary antibody in Tris-buffered saline containing 0.04% Tween 20; 1:100 dilutions for the rabbit polyclonal anti-EPO-R antibody (C20, Santa Cruz Biotechnology, Santa Cruz, CA), anti-EPO  
25 antibody (H-162, Santa Cruz Biotechnology, Santa Cruz, CA), and 1:1000 dilutions for mouse monoclonal anti-phosphorylated ERK1/ERK2 antibody (#9106S, New England Biolabs, Beverly MA). Blots were incubated for 1 hour with HRP-conjugated secondary antibody prior to the  
30 development using an ECL kit (Amersham). Our results demonstrate that both EPO and the EPO-Receptor (EPO-R) are expressed on the protein level in Langendorff perfused hearts (Fig. 2). Expression levels of both EPO and EPO-R appear unaffected by ischemia reperfusion and



by the application of EPO. In the next experiment, rat hearts were *in vivo* exposed to 10 U/ml EPO for 20 minutes. With the use of Western blotting we found an increase in a phosphorylated MAPK, notably ERK1 and to a lesser extent in ERK2 (Fig. 2).

In summary, the Western blot demonstrates the presence of EPO and its receptor in cardiac tissue. We found EPO-R mRNA in cardiac tissue, but were unable to detect EPO mRNA, suggesting that EPO is not locally produced.

Finally, we found EPO to change levels of phosphorylated MAPK, especially pERK-1, thus implying a functional role of EPO-R in cardiac tissue. This may have important implications for the application of EPO in heart failure, as the extracellular signal-regulated kinases pathway (ERK1/2) has been recognized as an important regulator of cardiac hypertrophy and myocyte survival in response to hypertrophic agonists and stress stimuli (Bueno and Molkentin, 2002).

#### *Immunohistochemistry*

To evaluate the EPO and EPO-R expression pattern in rat heart tissue, complete mid-ventricular myocardial slices were obtained from the control rat group. Tissue sections were fixed and paraffin-embedded. Histological slices of approximately 3  $\mu$ m were sectioned, dewaxed and rehydrated with graded ethanol. The sections were incubated with anti-EPO-R antibody (C20, Santa Cruz Biotechnology, Santa Cruz, CA) and with anti-EPO antibody (H-162, Santa Cruz Biotechnology, Santa Cruz, CA) using experimental methods well known to persons skilled in the art of immunohistochemistry. A two-step indirect peroxidase detection system was employed to visualize the expression pattern of EPO and EPO-R. All incubations were performed at room temperature and negative controls omitting the

primary antibody were performed simultaneously. Using these immunohistochemistry in non-ischemic rat heart tissue, EPO expression was found in a number of rats (n=4), where the EPO expression appeared to be limited to arterioles and capillaries. No EPO expression was found in cardiomyocytes or in fibrocytes. The expression of EPO-R was also mostly restricted to arterioles and capillaries, although the cardiomyocytes showed a weak staining for EPO-R.

These findings further emphasize a possible role of EPO and EPO-R in angiogenesis.

#### **Human heart**

Sections of formaline-fixed paraffin embedded human heart are obtained from routine autopsy cases (Dept. Pathology, Academic Hospital Groningen). Normal autopsy material harboring no cardiac pathology is obtained from at least 10 individuals. This material is used for Western blotting and immunohistochemistry as described above for the rat heart tissue.

#### **Example 2. Effect of EPO in acute ischemic events.**

The EPO-receptor (EPO-R) is found to be expressed at high concentrations in neuronal tissues (Digicaylioglu et al. 1995; Juul et al. 1997). The effects caused by (temporary) hypoxia due to cerebral ischemia may be mitigated by administering erythropoietin (EPO), as disclosed in WO 00/35475. Digicaylioglu and Lipton (2001) have shown that preconditioning with EPO protects neurons in ischemic injury models and prevents apoptosis. As disclosed herein, EPO and the EPO-R are also expressed in cardiac tissue. Cardiac tissue that is susceptible to hypoxia may therefore benefit from treatment with EPO (see also e.g. WO 00/61164, WO 01/82952).

Apoptosis and the release of purines from the heart are measured to determine the effect of EPO in circumstances in which the heart tissue becomes ischemic. For this, ischemic/reperfusion (I/R) experiments in

5 isolated rat hearts suspended in a so-called Langendorff apparatus (Van Gilst et al. 1988) are performed with and without the administration of EPO, using methods generally known to persons skilled in the art. The recombinant EPO is preferably obtained as described in WO

10 00/63403 using purification methods known to persons skilled in the art of protein production and isolation (see also PCT/NL02/00686). An alternative source of EPO is the commercially available epoetin alpha (Eprex). Four separate experimental groups are used, each comprising 8

15 Sprague Dawley (SD) rats. Each rat weighs approximately 250 grams. These groups are:

- SD rats without I/R, without EPO
- SD rats without I/R, with EPO
- SD rats with I/R, without EPO
- 20 - SD rats with I/R, with EPO

The rats are anaesthetized and the heart is rapidly excised. The aorta is immediately perfused retrogradely. Coronary flow (CF) is measured by a microprocessor, which

25 controls the perfusion pressure by adjusting the peristaltic perfusion pump. CF, heart rate (HR), and left ventricular peak pressure are monitored continuously and stored in a computer database. After equilibrating for 15 min, baseline parameters are measured. Ischemia is

30 induced by ligation of the left coronary artery for 15 min. Then, reperfusion is induced by releasing the ligature and the hearts are allowed to recover for 15 min.

Purine release from the heart has been shown to reflect myocardial damage (Van Jaarsveld et al. 1989). The coronary effluent dripping from the heart is collected for measurement of purines released by the myocardium. Baseline samples are collected after stabilization of the preparation, and coronary effluent is sampled after 15 min ischemia and after 15 min of reperfusion, and purines are measured by high-liquid performance chromatography (HPLC). The general trend is that initial purine values released from the coronary effluent from non-EPO-treated animals start off at higher values, while the decrease of purine over time appears to be slower, as compared to EPO-treated animals.

At the end of the experiments, hearts are weighed and a midpapillary slice from the left ventricle is cut out and fixed. The non-infarcted part of the heart (posterior wall, IV septum) is snap-frozen in liquid nitrogen. As described above, polyclonal antibodies against EPO and EPO-R are applied to determine the expression of both proteins.

Apoptosis is detected as follows. Sections from paraffin embedded tissue blocks are placed on coated slides for *in situ* detection of apoptotic cells. Nuclear DNA fragments are visualized by an enzymatic reaction, using the ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg USA) following the manufacturer's instructions. Number and distribution of stained cells, morphologic nuclear features and intensity of staining are evaluated.

30

### **Example 3. Effect of EPO in chronic ischemia model systems.**

Myocardial infarction is induced in rats and the role of EPO, which is administered *in vivo*, is determined

by measuring Left Ventricular Pressure (LVP), infarct size, apoptosis and microvascular density. For this, SD rats are either sham-operated (SH) or myocardial infarcted (MI) and treated with EPO (see above) in a concentration of 400 units per kg sc, or with saline, every day for 4 weeks. Four separate experimental groups are used, each comprising 8 SD rats. Each rat weighs approximately 250 grams. The used groups are:

- SD rats with sham operation, without EPO
- 10 - SD rats with sham operation, with EPO
- SD rats with myocardial infarction, without EPO
- SD rats with myocardial infarction, with EPO

The myocardial infarction model has been described elsewhere (Pinto et al. 1993). In brief, anaesthesia is induced and a left-sided thoracotomy is performed and MI is created by ligating the left coronary artery with a 6-0 silk suture, 1-2 mm after the bifurcation with the aorta. In sham-operated rats, the same operation will be executed, without ligating the suture.

The Left Ventricular (LV) function is determined as follows. After 4 weeks rats are anaesthetized and the right carotid artery is cannulated with a pressure transducer catheter. After a 3 min period of stabilization, maximal LVP, LV end-diastolic pressure (LVEDP) and heart rate are recorded. Hereafter, the catheter is withdrawn to measure systolic blood pressure in the aortic root. As indices of global contractility and relaxation, the maximal rates of increase and decrease in LVP (systolic  $dP/dt$  and diastolic  $dP/dt$ ) is determined, which will be further corrected for peak systolic LVP.

The infarct size is determined by histological analysis by staining for LDH using general methods known

to persons skilled in the art. Total epicardial and endocardial circumference of the left ventricle and epicardial and endocardial scar length of the infarcted area are determined by means of a computerized  
5 planimeter. Infarct size is calculated by dividing the sum of the scar lengths by the sum of the total circumferences, as previously described in detail (Pinto et al. 1993). Furthermore, apoptosis is measured as described above.

10 The microvascular density is determined as follows (Loot et al., 2002). The paraffin embedded LV slice is cut and stained with hematoxylin-eosin for histological analysis to calculate infarct size and with RECA-1  
15 antibody to visualize microvessels using methods known to persons skilled in the art. Microvessel density per mm<sup>2</sup> is measured in the spared myocardium (opposing the infarction, usually ventricular septum or posterior wall). From each rat, seven to ten microscopic high power  
20 fields with transversely sectioned myocytes are digitally recorded with appropriate software. The microcirculation is defined as vessels beyond the third order arterioles, with a diameter of 150 µm or less, supplying tissue between arterioles and venules. Myocyte surface areas are  
25 measured by morphometry, selecting myocytes with a central nucleus with the largest possible surface area with image analysis software (Loot et al., 2002).

**Example 4. Determination of EPO and EPO-R levels in chronic ischemia in human heart.**

30 The expression levels of EPO and EPO-R are determined by the level of mRNA, using a semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) technique. For this, total RNA is isolated using the acid guanidium thiocyanate lysis

method (Chomczynski and Sacchi 1987). The RNA is obtained from tissue from patients with ischemic heart failure. The tissue is removed during cardiac catheterization by right ventricular endomyocardial biopsy from the right jugular or femoral vein, using standard techniques known to persons skilled in the art. Reverse transcription of RNA and amplification of cDNA is performed by RT-PCR. The cDNA of interest and the cDNA of the housekeeping enzyme GAPDH are detected by real-time RT-PCR as described above.

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